POLYPHENOL OXIDASE IN THE TEGUMENTAL GLANDS IN RELATION TO THE MOLTING CYCLE OF THE ISOPOD CRUSTACEAN ARMADILLIDIUM VULGARE[1]

J. ROSS STEVENSON

Department of Biology, Kent State University, Kent, Ohio

Various hypotheses have been advanced to explain the function of the tegumental glands of Crustacea. The suggestions by early workers that they might be salivary glands, slime glands, sense organs, or excretory organs have been reviewed and disposed of by Yonge (1924, 1932), who believed that they might secrete the epicuticle. (Yonge called this layer the cuticle, but in this paper the terminology adopted by Richards, 1951, will be employed. According to this terminology, the outer, non-chitinous layer of the cuticle is the epicuticle and the inner chitinous layer is the procuticle.) Drach (1939), on the other hand, thought the epidermis secretes the epicuticle. Arguments for and against Yonge's view have been reviewed by Dennell (1947b, 1960), who suggested that since the tegumental glands are most abundant where the cuticle is tanned the most, they may produce the polyphenol oxidase involved in tanning.

This suggestion by Dennell has been investigated by Krishnan (1951). He used the nadi reagent as a test for oxidase and obtained a positive reaction in the tegumental glands of *Carcinus*, their ducts, and the pigment layer of the cuticle during middle and late intermolt when melanin is forming in the pigment layer. The reaction was thermo-labile and cyanide-sensitive. Also the glands and their ducts became dark brown in catechol during this same period. He concluded the tegumental glands secrete polyphenol oxidase for melanin production in the cuticle. Whether they also secrete polyphenol oxidase earlier for tanning is not known. Apparently Krishnan did not study this earlier stage in the molting cycle.

In order to discover whether the tegumental glands secrete polyphenol oxidase for tanning, the molting cycle of the pillbug, *Armadillidium vulgare*, has been studied and polyphenol oxidase has been looked for in the tegumental glands of the legs at every stage of the molting cycle.

Materials and Methods

Several hundred specimens of *Armadillidium vulgare* collected in a garden in Seattle, Washington, were maintained in the laboratory in large jars partly filled with moist dirt. Pieces of carrot and potato were placed in each jar for food.

Tegumental glands were obtained for study by dissection of pereiopods in 0.35 *M* NaCl. A separate leg was removed from a living animal for each test and used immediately. The glands of one segment were tested with the reagent and

[1] This investigation was supported in part by research grant B-2045 (C1) from the National Institutes of Health, Public Health Service.

the glands of another segment of the same leg were tested with the reagent plus inhibitor. The glands are very abundant in the carpus and merus, less abundant in the ischium, and small and scarce in the basis. They seem to be absent from the propus and dactylus. To ensure uniformity, only animals in molting stage C were used for the tests for polyphenol oxidase. The dissections were made in 0.35 *M* NaCl because this concentration has a freezing point depression close to 1.18°, the average value for *Armadillidium* according to Parry (1953). No visible changes in the glands take place in this solution, whereas the secretory droplets in the glands burst in hypotonic solutions.

The nadi reagent was prepared immediately before use by mixing 0.1 ml. of 1% a-naphthol in 40% ethanol and 0.1 ml. of 1% N,N-dimethyl-p-phenylenediamine hydrocholoride with 2.5 ml. of buffer. Acetate, phosphate, carbonate, and glycine buffers were used to provide a range in pH from 4 to 11.

A stock of *Armadillidium vulgare* adults was also maintained, one animal to a jar, in 100-ml. jars, each having a shallow layer of moist dirt and potato and carrot for food. By making daily observations on each animal, criteria for recognizing the different molting stages were developed, as described below.

Results

Molting stages. The stage designations developed by Drach (1939) for the Decapoda and some of the modifications of these designations developed by Charniaux-Legrand (1951) for the Amphipoda have been used.

The half of the body posterior to the suture separating the fourth and fifth free thoracic segments molts first, and the half of the body anterior to this suture molts a few days later. Since the molting stages of the two halves of the body can be determined independently, one can designate the molting stage of an animal by stating the stages of each half; for example, anterior: stage C^a, posterior: stage $D_1{}^p$. The letters a and p indicate anterior and posterior.

The following descriptions apply to either half.

Stage A. Immediately after the molt. The body half appears dark brown or black, and the cuticle is soft, shiny, and slightly sticky. Grains of dirt may adhere to it. The legs are not functional, but the animal can walk with the legs of the other half of the body. At first the half in stage A is only about half as long as the other half, but it begins to expand immediately. At the end of stage A, expansion is completed, the cuticle is becoming gray in color, and the legs are beginning to be functional. Duration of stage A: 1–2 hours.

Stage B. The cuticle is gray in color. It has only begun to harden; the terga can be depressed without cracking. The body half has expanded to its full, new size. The pereiopods are functional but their cuticle, as measured in optical section, has not yet reached its definitive thickness (Fig. 1). The animal normally eats its exuviae during this stage. Duration of stage B: several hours.

Stage C. The body has achieved its definitive coloration and hardness. In the early part of this stage the cuticle of the pereiopods becomes progressively harder, beginning distally. Stage C could be subdivided in terms of this progressive hardening. At the same time the cuticle of the pereiopods becomes progressively thicker (compare Figs. 1 and 2). Duration of stage C: about 15–60 days.

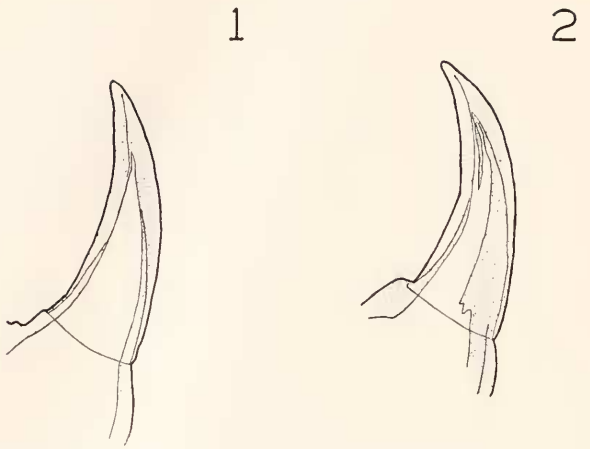

FIGURE 1. Tip of left first pereiopod in stage B. 280 ×.
FIGURE 2. Tip of right first pereiopod of same specimen 10 days later. Stage C.

Stage D. Preparation for the molt. This stage can conveniently be subdivided, as follows:

D_1. New claws begin to form in the pereiopods, as described by Charniaux-Legrand (1951) in amphipods (Fig. 3). While the posterior half of the body is in stage D_1 a pair of white plates develops on each of thoracic sterna 1 to 4. Duration of stage D_1: a few days to a week.

D_2. The new claws have become amber-colored, and the pre-exuvial layers of the new cuticle are secreted over the entire body half. While the anterior half of the body is in stage D_2 the white sternal plates disappear. Duration of stage D_2: about one day.

D_3. As in amphipods, nothing corresponding to stage D_3 of decapods is readily discernible in *Armadillidium*.

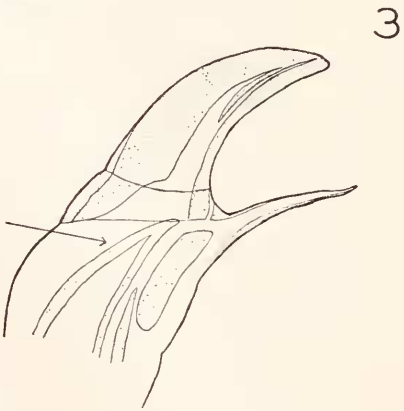

FIGURE 3. Tip of right first pereiopod in stage D_1. The tip of the new dactylus (arrow) can be seen inside the old. The leg was too opaque for the proximal part of the new dactylus to be seen.

D_4. The body half can be compressed easily with the fingers, and the old cuticle can be flaked off. Duration of stage D_4: a few hours.

Stage E. Ecdysis. Molting may occur at any hour, day or night.

Nadi test at different stages of the molting cycle. The tegumental glands of the legs were tested with the nadi reagent in phosphate buffer at pH 7.4 in every stage of the molting cycle and at different intervals within stages C and D. In every case, deep blue color developed in the secretory droplets within a few minutes in the absence but not in the presence of 0.002 *M* NaCN.

Additional tests for polyphenol oxidase. In addition to NaCN, two other inhibitors, NaN_3 and salicylaldoxime, were used with the nadi reagent, and the effect of heat was tested. According to Keilin (1936), catechol oxidase (polyphenol oxidase) from mushrooms was not inhibited by 0.002 *M* NaN_3 at pH 7.3, but 68% inhibition was obtained at pH 5.9. On the other hand only 0.001 *M* NaN_3 produced 80–90% inhibition of cytochrome oxidase at pH 6.5–7.5. In order to test for this same kind of behavior by the oxidase of the tegumental glands,

TABLE I

Results of nadi tests on tegumental glands at different pHs

Without inhibitor										
pH 4	5	6	6.8	7	7.2	7.4	8	9	10	11
−	−	+	+	+	+	+	+	+	±	±
With 0.02 *M* NaN_3										
pH	5	6	6.8	7	7.2	7.4	8	9	10	11
	−	−	−	−	+	+	+	+	±	−

the nadi reagent in 0.002 *M* NaN_3 was used in buffers over a pH range from 4 to 11. For comparison with cytochrome oxidase, the nadi-positive granules of the leg muscles of *Armadillidium* were used. The two tissues could be examined together on the same slide. It was found that 0.002 *M* NaN_3 completely prevented the development of blue color in the granules of the muscles throughout the entire range, pH 6 to pH 8, in which indophenol blue developed in muscles in the control slides. The same concentration of NaN_3 had no discernible effect on the reaction in the tegumental glands at any pH, however. Accordingly, the series was repeated using 0.02 *M* NaN_3. This time, inhibition was obtained in the secretory droplets of the tegumental glands below pH 7.2, as shown in Table I. Without azide, blue color developed in all tests from pH 6 to pH 9. At pH 10 and pH 11, blue color sometimes developed in some of the glands. With 0.02 *M* NaN_3, no blue color developed at pH 5, 6, 6.8, or 7, and normal blue color developed as rapidly as in the controls at pH 7.2, 7.4, 8, and 9. Blue color sometimes developed in some of the glands at pH 10, never at pH 11. A pH of 4 was not tested.

Salicylaldoxime is a reagent for copper and therefore should inhibit the copper-containing enzyme polyphenol oxidase. The tegumental glands were tested with

the nadi reagent with and without 0.001 *M* salicylaldoxime and with and without 0.01 *M* salicylaldoxime. At 0.001 *M*, blue color developed about half as rapidly (14–15 minutes) as in the controls (5–6 minutes). At 0.01 *M*, no color developed at all.

The nadi test was also negative in glands that had been incubated at 65° C. for 30 minutes.

Fresh tegumental glands were also treated with 1% 3,4 DL dihydroxyphenylalanine (dopa) in phosphate buffer, pH 6.8. After three hours at 22° C., the secretory droplets were black. None of the other tissues had become colored.

Discussion

Studies on molting and related phenomena in crustaceans are sometimes hampered by the difficulty of obtaining the desired stages in the molting cycle when needed. By using *Armadillidium* and the criteria for recognizing the different stages as described in this paper, however, one can obtain any stage at any time. For instance, in a stock of only 60 animals an average of one will molt each day. To designate short periods within each of the primary stages, substages or number of hours before or after an event can be used. George and Sheard (1954) have used the latter method in their description of changes in the epidermis and cuticle of *Porcellio scaber*.

From the evidence presented in this paper, the tegumental glands seem definitely to contain polyphenol oxidase. The nadi test is rather uncertain and variable—poor staining was obtained if the stock solutions were more than a month or less than a week old! But inhibition of the reaction by cyanide, azide, salicylaldoxime, and heat clearly indicates the presence of an enzyme. Inhibition by azide was exactly as predicted from Keilin's work with catechol oxidase of mushrooms, except that a higher concentration of inhibitor was needed.

The most specific test for polyphenol oxidase used was treatment with dopa, which is oxidized to dopa quinone by this enzyme. The dopa quinone then becomes melanin by polymerization.

The tegumental glands of the legs of *Armadillidium* are similar histologically to tegumental glands of other parts of the body and to tegumental glands of other crustaceans. Their function is probably to secrete polyphenol oxidase into the epicuticle from where it diffuses inward and catalyzes sclerotization in the epicuticle and exocuticle (Dennel, 1947b). The evidence for this is three-fold. First, the glands develop and degenerate with each molting cycle, and the peak in their activity occurs some time before molting (Yonge, 1932; Gorvett, 1946). It may be that the peak coincides with the appearance of polyphenol oxidase in the epicuticle, but further work will have to be done to determine whether this is the case. Second, the ducts of the glands extend to or through the epicuticle, and these ducts are most abundant where the cuticle is tanned the most (Dennell, 1947b, 1960). The presence of the ducts extending through the procuticle to the epicuticle is significant because the epicuticle is the part of the cuticle where polyphenol oxidase appears first (Dennell, 1947b). However, in the insect *Sarcophaga,* in which the enzyme also appears first in the epicuticle, there is evidence that it originates in the epidermis, diffuses through the procuticle, and somehow accumulates in the epicuticle (Dennell, 1947a). Third, as demonstrated in this paper, the tegu-

mental glands do contain polyphenol oxidase. In addition to this evidence, direct evidence that the glands release polyphenol oxidase into the epicuticle at or before the time when it is used in tanning would be desirable before one concluded that they do so. It may be that the enzyme is involved in the metabolism of the glands and that the secretory product is some other material. Both mucus (Farkas, 1927) and lipid (Yonge, 1932) have been reported in tegumental glands, and there is evidence that tyrosinase (polyphenol oxidase) may function as a terminal oxidase in respiration, at least in plants and insects (Dawson and Tarpley, 1951; Patterson, 1958).

The presence of polyphenol oxidase in the tegumental glands of the legs of *Armadillidium* throughout the molting cycle is not inconsistent with the idea that the enzyme is released at a particular time. The enzyme may be in storage most of the time. However, it raises the question of why *Carcinus* is different in this respect. Krishnan found polyphenol oxidase only during middle and late intermolt in *Carcinus*. Another difference in *Carcinus* is that the tegumental glands also apparently secrete polyphenol oxidase into the pigment layer, where it catalyzes melanin production (Krishnan, 1951). Presumably this does not occur in *Armadillidium,* which lacks a pigment layer.

Undoubtedly some of the different types of tegumental glands perform different functions. Gorvett (1946) has described six types in isopods, and he presents evidence that the lobed glands of the uropods and lateral plates secrete bad-tasting and bad-smelling material that protects against spiders (Gorvett, 1951, 1952, 1956). Other types may have yet different functions.

Summary

1. Criteria are presented for distinguishing the molting stages of *Armadillidium vulgare*.

2. An oxidase is present in secretory droplets in the tegumental glands of the legs throughout the entire molting cycle.

3. This oxidase seems to be the polyphenol oxidase involved in sclerotization of the cuticle.

LITERATURE CITED

Charniaux-Legrand, H., 1951. Le cycle d'intermue des amphipodes et ses particularités chez les formes terrestres (Talitridae). *Arch. Zool. Exp.*, **88**: 178–204.

Dawson, C. R., and W. B. Tarpley, 1951. Copper oxidases. Chapter 57 *in:* The Enzymes, vol. 2, pt. 1. Academic Press Inc., N. Y.

Dennell, R., 1947a. A study of an insect cuticle: the formation of the puparium of *Sarcophaga falculata* Pand. (Diptera). *Proc. Roy. Soc. Lond., Ser. B,* **134**: 79–110.

Dennell, R., 1947b. The occurrence and significance of phenolic hardening in the newly formed cuticle of Crustacea Decapoda. *Proc. Roy. Soc. Lond., Ser. B,* **134**: 485–503.

Dennell, R., 1960. Integument and exoskeleton. Chapter 14 *in:* The Physiology of Crustacea, vol. 1, Metabolism and Growth. Academic Press, N. Y. and Lond.

Drach, P., 1939. Mue et cycle d'intermue chez les crustacés décapodes. *Ann. Inst. Océanogr.*, **19**: 103–391.

Farkas, B., 1927. Zur Kentnis der Tegmentaldrüsen der Dekapoden. *Zool. Jahrb. Abt. Anat. Ontog. Tiere,* **49**: 1–56.

George, R. W., and K. Sheard, 1954. Ecdysis in the isopod *Porcellio scaber* (Latreille). *Australian J. Zool.*, **2**: 75–85.

Gorvett, H., 1946. The tegumental glands in the land Isopoda. A. The rosette glands. *Quart. J. Micr. Sci.*, **87**: 209–235.

Gorvett, H., 1951. The tegumental glands in the land Isopoda. B. The lobed glands: structure and distribution. *Quart. J. Micr. Sci.*, **92**: 275–296.

Gorvett, H., 1952. The tegumental glands in the land Isopoda. C. The lobed glands: the properties of their secretion and their mode of action. *Quart. J. Micr. Sci.*, **93**: 17–29.

Gorvett, H., 1956. Tegumental glands and terrestrial life in woodlice. *Proc. Zool. Soc. Lond.*, **126**: 291–314.

Keilin, D., 1936. The action of sodium azide on cellular respiration and on some catalytic oxidation reactions. *Proc. Roy. Soc. Lond., Ser. B*, **121**: 165–173.

Krishnan, G., 1951. Phenolic tanning and pigmentation of the cuticle in *Carcinus maenas*. *Quart. J. Micr. Sci.*, **92**: 333–342.

Parry, G., 1953. Osmotic and ionic regulation in the isopod crustacean *Ligia oceanica*. *J. Exp. Biol.*, **30**: 567–574.

Patterson, D. S. P., 1958. Succinate as a respiratory inhibitor in *Tenebrio molitor* pupal-tissue preparations. *Biochem. J.*, **68**: 225–231.

Richards, A. G., 1951. The Integument of Arthropods. University of Minnesota Press, Minneapolis.

Yonge, C. M., 1924. Studies on the comparative physiology of digestion. II. The mechanism of feeding, digestion, and assimilation in *Nephrops norvegicus*. *J. Exp. Biol.*, **1**: 343–389.

Yonge, C. M., 1932. On the nature and permeability of chitin. I. The chitin lining the foregut of decapod Crustacea and the function of the tegumental glands. *Proc. Roy. Soc. Lond., Ser. B*, **111**: 298–329.